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Research paper

Identification and expression profile of multiple genes in channel catfish fry 10 min after modified live *Flavobacterium columnare* vaccination

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ABSTRACT

Using PCR-select subtractive cDNA hybridization technique, 32 expressed sequence tags (ESTs) were isolated from 96 clones of a channel catfish (*Ictalurus punctatus*) fry subtractive library 10 min post-vaccination with a modified live *Flavobacterium columnare* vaccine. The transcription levels of the 32 ESTs in response to *F. columnare* vaccination were then evaluated by quantitative PCR (QPCR). Of the 32 ESTs, 28 were upregulated in at least one vaccinated fish. Of the 28 upregulated ESTs, 12 were consistently induced at least 2-fold higher in vaccinated fish compared to unvaccinated control fish. Of the 12 upregulated genes, three (triglyceride lipase, PIKK family atypical protein kinase, and CCR4-NOT transcription complex subunit 1) were consistently upregulated greater than 3-fold. The 12 consistently upregulated genes also included CD59, polymerase (RNA) I polypeptide C, pyrophosphatase (inorganic) 1, mannose-P-dolichol utilization defect 1, nascent polypeptide-associated complex subunit alpha, hemoglobin-beta, fetuin-B, glyoxalase domain containing 4, and putative histone H3. The 28 upregulated ESTs represent genes with putative functions in the following five major categories: (1) immune response (46%); (2) signal transduction (21%); (3) transcriptional regulation (11%); (4) cell maintenance (11%); and (5) unknown (11%).

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1. Introduction

Flavobacterium columnare is a bacterial pathogen of freshwater fish worldwide, causing skin lesions, fin erosion and gill necrosis known as columnaris disease. In the United States, columnaris disease causes annual losses of millions of dollars for the channel catfish (*Ictalurus punctatus*) industry (Wagner et al., 2002; Shoemaker et al., 2007). Columnaris disease has also become the most serious threat to salmon (*Salmo salar*), smolt trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) production during the last two decades in Finland, where intensive fish farming is practiced (Pulkkinen et al., 2010). To control columnaris disease, live attenuated *F. columnare* vaccines have been developed to protect channel catfish (Shoemaker et al., 2005, 2007). It has been reported that the modified live *F. columnare* vaccine (formerly ARS *F. columnare* B-30303, currently licensed to Intervet/Schering Plough, Millsboro, DE as Aquavac-COL™) is protective against columnaris disease in channel catfish fry in lab studies, with relative percent survival (RPS) values exceeding 96% (Shoemaker et al., 2005). In recent field trials, largemouth bass fry (*Micropterus salmoides floridanus*) vaccinated with Aquavac-COL has shown a 43% lower risk of death (Bebak et al., 2009). However, it is currently not clear how this vaccine induces immune responses in catfish, therefore resulting in disease protection.

To understand how Aquavac-COL works at the molecular level without any preconception, we used PCR-select

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suppression subtractive hybridization in this study. Since its first introduction to researchers (Diatchenko et al., 1996), suppression subtractive hybridization has been widely used by researchers to understand fish innate immunity (Dios et al., 2007; Zhang et al., 2007a,b; Pridgeon et al., 2009a) and other molecular studies (Singh et al., 2008; Zhao et al., 2008; Zhou et al., 2008; Pridgeon et al., 2009b) because this technique does not require any previously known genome information for the organism (Sternberg and Gepstein, 2007; Hillmann et al., 2009). We used quantitative PCR (qPCR) to compare the transcriptional levels of genes in vaccinated fish and unvaccinated fish because qPCR has tremendous sensitivity and requires little or no post-amplification processing (Wong and Medrano, 2005). Furthermore, qPCR is a highly reproducible technique for the quantification of mRNA (Ashton and Headrick, 2007). Recently, 57 different genes have been isolated from a channel catfish anterior kidney 48 h after modified live *Edwardsiella ictaluri* vaccination (Pridgeon et al., 2009a). However, it is not clear whether channel catfish fry will respond similarly to a modified live vaccine of *F. columnare* as that to the live *E. ictaluri* vaccination. The modified live vaccine of *F. columnare* is usually used to immerse channel catfish fry for 10 min. Therefore, the purpose of this study is to use PCR-select subtractive cDNA hybridization technique to identify genes in channel catfish fry induced by the modified live vaccine of *F. columnare* at 10 min post-vaccination.

2. Materials and methods

2.1. Experimental fish, vaccine strain, and vaccination protocol

Channel catfish eggs (Industry pool strain) were obtained from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS and maintained at the USDA-ARS-Aquatic Animal Health Unit at Auburn, AL. Fish were hatched and maintained in dechlorinated city water in 340 l tanks to ensure that the catfish fry remained naïve to *F. columnare*. Catfish frys (7–10 days post-hatch) were used for vaccination and were 60 ± 9 mg at time of vaccination. Fish were vaccinated with Aquavac-COL™ following the manufacturer's protocol (Intervet/Schering Plough, Millsboro, DE). Briefly, one vial of the vaccine was thawed at 26 °C in a water bath prior to dilution (1 ml of vaccine in 18.9 l of water) at 26 ± 2 °C. Three channel catfish frys were transferred into the prepared vaccine bath provided with aeration through air stones via an air blower and held for 10 min. As sham-vaccination controls, three channel catfish frys were transferred into the prepared sham-vaccination bath as described above except no vaccine was added to the water. After the 10-min exposure time, the fish frys (both vaccinated and non-vaccinated) were flash-frozen on dry ice and stored at −80 °C until RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from whole channel catfish fry using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All total RNAs were quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Total RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C. The first strand cDNAs used for quantitative PCR were synthesized using AMV reverse transcriptase (Invitrogen, Carlsbad, CA). For subtractive library construction, total RNA were pooled from the three vaccinated or non-vaccinated fish. cDNAs were then synthesized from the pooled total RNAs using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA) as described by the manufacturer. The cDNAs that contained specific transcripts were referred to as "testers" (i.e. from vaccinated fish) and the reference cDNAs were referred to as "drivers" (i.e. from non-vaccinated fish). The double-stranded cDNAs of both testers and drivers were digested with RsaI to create smaller blunt-ended fragments to be used as testers or drivers according to the manufacturer's instruction (Clontech, Palo Alto, CA). The tester cDNAs were then subdivided into two portions (A and B) and modified by ligating with cDNA adaptors 1 and 2 (provided in the Kit), respectively.

2.3. Construction of subtractive cDNA library

Two-step subtractive hybridizations were performed according to procedures used previously (Pridgeon and Liu, 2003). Briefly, in the first step hybridization, two primary hybridization reactions (A and B) were formed by adding excess amounts of unmodified driver cDNA to separate portions A and B of tester cDNA samples at a 50:1 ratio. The samples were denatured for 2 min at 98 °C and allowed to anneal for 8 h at 68 °C. The remaining single-stranded, adaptor-ligated tester cDNAs were substantially enriched in each hybridization reaction for overexpressed sequences because non-target cDNAs present in the tester and driver formed hybrids. For the second step hybridization, A and B primary hybridization reaction solutions were mixed together without denaturing. These new hybrids were double-stranded tester molecules with different 5′-ends corresponding to the sequences of two different adaptors. Freshly denatured driver DNA was added to the reaction without denaturing the subtraction mix to further enrich new double-stranded tester molecules that are differentially expressed. After filling in the adapter ends with DNA polymerase, overexpressed sequences (tester cDNA) had different annealing sites on their 3′- and 5′-ends. The molecules were then subjected to suppression subtraction PCR as described by the manufacturer (Clontech, Palo Alto, CA). The PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were then transformed into One Shot® TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were then plated on Luria-Bertani (LB) plates containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (40 μg/ml).

2.4. Plasmid DNA isolation and sequencing

From the library, a total of 96 white colonies were subsequently picked to grow overnight in LB broth in the presence of ampicillin (100 μg/ml) in the Innova™ 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ)

at 37 °C and 235 rpm settings, respectively. Plasmid DNAs were isolated with QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). Plasmid DNAs were then digested by EcoRI at 37 °C for 1 h and subjected to 1% agarose gel electrophoresis. Plasmid DNAs that contained inserts were then sent to USDA-ARS Mid South Genomic Laboratories in Stoneville, MS for sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were then analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

2.5. Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). QPCR was performed using Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation of cDNA amount. All QPCR was performed on an Applied Biosystems 7000 Real-Time PCR System (ABI, Foster City, CA) using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 μl. The QPCR mixture consisted of 1 μl of cDNA, 0.5 μl of 5 μM gene-specific forward primer, 0.5 μl of 5 μM gene-specific reverse primer and 10.5 μl of 1× SYBR Green SuperMix. The QPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycle of 95 °C for 15 s and 60 °C for 1 min. All QPCR was run in duplicate for each cDNA sample and three fish cDNA samples were analyzed by QPCR.

2.6. QPCR data analysis

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (C_t) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: $\Delta C_t = C_t$ (sample) $- C_t$ (calibrator). Primers used for the amplification of the 18S rRNA gene was 18S-F (5′-ATGGCCGTTCTTAGTTGGTG-3′) and 18S R (5′-TAGGTAGCACACGCTGATCG-3′). The two primers were designed based on channel catfish 18S small subunit ribosomal RNA gene sequence (GenBank accession no. BE469353). The relative expression level of the specific gene in *F. columnare* vaccinated fish compared to that in non-vaccinated fish was then calculated by the formula $2^{\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t$ (vaccinated) $- \Delta C_t$ (non-vaccinated) as described previously (Pridgeon et al., 2009a). Data were analyzed by analysis of variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA).

3. Results

3.1. Characteristics of the subtractive cDNA library

A total of 96 clones were obtained from the whole fry subtractive library. Of the 96 clones, 53 contained inserts. Sequencing results revealed that these 53 ESTs represented 32 different genes (Table 1). All ESTs listed in Table 1 have been deposited in the GenBank EST database under accession numbers GW392457–GW392488. The most frequently detected ESTs were CNOT-1 and sodium/potassium-transporting ATPase, alpha subunit ($n = 3$). Of the 57 ESTs identified from the subtractive library, 10 shared homology with deposited *Danio rerio* proteins, three shared homology with deposited *I. punctatus* proteins, and two shared homology with deposited *S. salar* proteins (Table 1). The biggest insert size was 584 bp (GW392461) and the smallest insert size was 112 bp (GW392460). The average insert size of the 57 ESTs was 283 bp (Table 1).

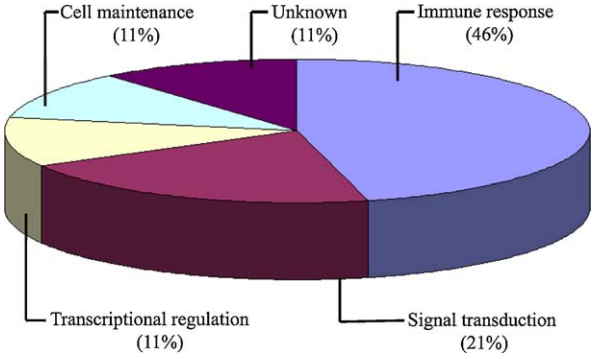

Fig. 1. Classification of the differentially expressed genes identified from the subtractive library. Pie charts representing the distribution of the 28 upregulated genes according to their putative biological function.

3.2. Expression profiling of the 32 ESTs in catfish fry 10 min after F. columnare vaccination

To determine whether the transcriptional levels of the 32 ESTs isolated from the subtractive library were upregulated in *F. columnare* vaccinated catfish fry, gene-specific primers (Table 2) for the 32 ESTs were designed for relative qPCR experiments. qPCR results revealed that 12 ESTs were consistently induced at least 2-fold higher in all three vaccinated fish compared to unvaccinated control fish (Table 3). Of the 12 upregulated genes, three (triglyceride lipase, PIKK family atypical protein kinase, and CCR4-NOT transcription complex subunit 1/CNOT1) were consistently upregulated greater than 3-fold. The 12 consistently upregulated genes also included CD59, polymerase (RNA) I polypeptide C, pyrophosphatase (inorganic) 1, mannose-P-dolichol utilization defect 1, nascent polypeptide-associated complex subunit alpha, hemoglobin-beta, fetuin-B, glyoxalase domain containing 4, and putative histone H3. Of the 32 ESTs identified, 28 ESTs were induced at least 2-fold by *F. columnare* vaccination in at least one vaccinate (Table 3).

3.3. Classification of the 28 F. columnare vaccination-upregulated genes

The 28 ESTs upregluated genes were classified in terms of their putative functions (Table 4). The greatest portion (46%, Fig. 1) of the upregulated genes were involved in immune responses, including putative histone H3, triglyc-

Table 1
List of genes isolated from the *F. columnare* vaccinated vs. non-vaccinated catfish fry subtractive cDNA library.

No.	Accession no.[a]	Protein homology	Protein accession no.[b]	Organism	Identities (%)	*e* value	Score bits	Insert size(bp)
6	GW392457	Putative histone H3	CAZ34873	*Schistosoma mansoni*	100	4.00E−08	60.8	128
9	GW392458	Triglyceride lipase	BAB85636	*Anguilla japonica*	64	6.00E−37	156	341
11	GW392459	Beta-actin	ACR83576	*Danio rerio*	100	1.00E−27	125	178
16	GW392460	Glyoxalase domain containing 4	NP_001004613	*Danio rerio*	80	6.00E−10	67	112
17	GW392461	Fetuin-B	ABA33614	*Carassius auratus gibelio*	55	7.00E−21	103	584
19	GW392462	Hemoglobin-beta	AAR25199	*Ictalurus punctatus*	100	4.00E−21	103	148
20	GW392463	Nascent polypeptide-associated complex subunit alpha	ACQ58397	*Anoplopoma fimbria*	100	9.00E−08	59.7	135
24	GW392464	Unknown	Unknown	–	–	–	–	–
25	GW392465	La ribonucleoprotein domain family, member 1	XP_001920902	*Danio rerio*	100	1.00E−08	62.8	121
33	GW392466	Intelectin 2	ABW07847	*Ictalurus punctatus*	100	5.00E−64	246	376
37	GW392467	pyrophosphatase (inorganic) 1	CAX14788	*Danio rerio*	73	7.00E−37	156	341
38	GW392468	Polymerase (RNA) I polypeptide C	CAM12918	*Danio rerio*	91	1.00E−74	281	456
39	GW392469	Similar to CCR4-NOT transcription complex subunit 1/CNOT1	XP_001599430	*Nasonia vitripennis*	33	2.50E+00	35	217
41	GW392470	C1R/C1S subunit of Ca2 + -dependent complex	NP_001117852	*Oncorhynchus mykiss*	70	7.00E−35	149	287
42	GW39247 1	PIKK family atypical protein kinase	XP_001316510	*Nakamurella multipartita*	48	5.50E+00	33.9	285
45	GW392472	Membrane-spanning 4-domains subfamily A member 8A	NP_001135067	*Salmo salar*	77	2.00E−16	88.6	205
46	GW392473	Splicing factor 3b, subunit 1 isoform 1	AAI72216	*synthetic construct*	98	3.00E−51	204	309
47	GW392474	Similar to Tyrosine aminotransferase	XP_001367424	*Monodelphis domestica*	83	2.00E−20	102	168
48	GW392475	Matrix metalloproteinase	NP_001117671	*Oncorhynchus mykiss*	60	2.00E−34	148	505
50	GW392476	Novel protein similar to vertebrate receptor accessory protein family	CAX13095	*Danio rerio*	87	5.00E−14	80.5	123
56	GW392477	Unknown	Unknown	–	–	–	–	–
67	GW392478	Myozenin	ACP30433	*Tetraodon nigroviridis*	72	7.00E−16	86.7	176
69	GW392479	CD59	ABI18969	*Ictalurus punctatus*	96	2.00E−28	128	326
70	GW392480	sodium/potassium-transporting ATPase, alpha subunit	CAA53714	*Anguilla anguilla*	95	1.00E−45	185	273
75	GW392481	unknown	unknown	–	–	–	–	–
80	GW392482	RIO kinase 3	NP_001003614	*Danio rerio*	88	1.00E−69	265	427
82	GW392483	Muscle creatine kinase b	NP_001099153	*Danio rerio*	96	7.00E−08	60.1	377
83	GW392484	Actinin alpha 3	ACH85265	*Tetraodon nigroviridis*	95	1.00E−27	125	187
85	GW392485	Galectin-4	NP_001140054	*Salmo salar*	52	3.00E−09	64.7	175
86	GW392486	Neurotoxin/C59/Ly-6-like protein	AAR20998	*Ctenopharyngodon idella*	69	1.00E−13	79.3	280
89	GW392487	Microtubule-associated protein, RP/EB family, member 2	NP_001103172	*Danio rerio*	95	3.00E−13	78.2	240
90	GW392488	Similar to myosin binding protein C, fast-type isoform 2	XP_863363	*Danio rerio*	76	4.00E−05	50.8	200

[a] EST accession number deposited at NCBI GenBank.
[b] Protein accession number at NCBI GenBank.

Table 2
Gene-specific primers used in qPCR.

Clone no.	Accession no.[a]	Forward primer (5′–3′)	Reverse primer (5′–3′)
6	GW392457	ATTCATGCCAAGAGGGTCAC	AAGATTAAAACAAGACAAAATGGTCA
9	GW392458	GCCCAATGGAGTGGATAAGA	GCGTGCCATTGTAATTCTGA
11	GW392459	CCTTACGGATATCCACATCACA	TATGAGCTGCCTGACGGACA
16	GW392460	ACGGAAGCACCTGGTGGATA	AACGCTGCAGGTCAGACAC
17	GW392461	GAAGGTGAGACACACGAGCA	GTCGGGTTTCTCATGCTGAT
19	GW392462	GGAACTTCTGCCAAGTCTCG	CAGCAACTTCACGCTTCTTG
20	GW392463	TCGTTGTTGTTATTCTTCAGTGC	CGAAGAGGAAGAGGCTGATG
24	GW392464	GCTACGTTTCCGAGTGTGGT	AGCCTGTGTCCTTGCTTTGT
25	GW392465	CAATGAGAGGAAACGGGTTG	CATCTTCCTGTTGAAGTTGTCTC
33	GW392466	AGCGGACCTTCTATCCCAGT	CTGTCTTCAGGAAGCCCAAC
37	GW392467	GCCAGTTCAGGAGGTCTGAG	GATTGGGCCTTTTCTTGTCA
38	GW392468	CATGCAAAGTTTTCCCCAGT	CCTTAGCCACCTGTTTTCCA
39	GW392469	TGCTCTTTTTGCATGAGCAC	AGGACCAAAGCTGACCCTTT
41	GW392470	TCGAATCACTCCCAATTTCC	CAGTAACGCCTCCGGTTCT
42	GW39247 1	AAAGGGGTCATTTGAAGCTG	TCAATCAAGGCGTTTTAGGC
45	GW392472	TTTGTGCCAAATCCACTGAA	ATCGGTGATCTTGGTCTTGC
46	GW392473	GAAGCAGTGCTGTGGAACAA	ATTTCTGCTGCACCGACTTT
47	GW392474	CTTGAGGCAACTGAGAGGATG	GCCAGAGAAATCCTGGGAGA
48	GW392475	CGCAAGAGTCTCGGTAGCTT	AGGTGAAACCGTTGTTCTGG
50	GW392476	CAACCACTGTGTGTCGTCCT	ATGGTGCATCTCTCCTCTGC
56	GW392477	TCTCAATGCCATCGTATCCA	CCCTGCAGTGACCACATCTT
67	GW392478	GGATCTGCCCCAATACAAGA	GATGGTCGAGAGTCGATGTG
69	GW392479	CAGGGTGCAGGATGAAAGTT	AGTATCAGGCAGCCGTCATT
70	GW392480	GTAATTGTGCAATGGGCTGA	CCAGCTTGGTTTCAATGGAT
75	GW392481	TCGACCAACACACCACTGAT	AACTGAAGCGCAAACCATTT
80	GW392482	CTTAGGAGCTGGCACATGGT	GAATTCAAGAACCGGGACAA
82	GW392483	GCGATTTGATCCCAAGATGT	CCTGCCCAGAAGTAAAGTGG
83	GW392484	GGACAACCTGGGCACTCTAA	ATGTCCTGCAGATCCTCCAT
85	GW392485	TGTTGACGTCTCCATAAATGTG	TCCCATTTCCAAAGGATCAG
86	GW392486	CCTGCGGCTATAAGCAAGAC	TCATGGTCCAGCACTTTCCT
89	GW392487	AAAAGCCTAGGTTGTCGTCTG	TGTTGGTTCCATTGTCCTGA
90	GW392488	CAGCAAACGGTGTCTTCAAA	AGGCCATCAACGAATATGGT

[a] EST accession number deposited at NCBI GenBank.

eride lipase, glyoxalase domain containing 4, fetuin-B, hemoglobin-beta, nascent polypeptide-associated complex subunit alpha, intelectin 2, pyrophosphatase (inorganic) 1, C1R/C1S subunit of Ca^{2+}-dependent complex, membrane-spanning 4-domains subfamily A member 8A, tyrosine aminotransferase, matrix metalloproteinase, and CD59. The second largest portion (21%, Fig. 1) of the upregulated genes were related to signal transduction, including PIKK family atypical protein kinase, RIO kinase 3 (RIOK3), muscle creatine kinase b, splicing factor 3b subunit 1 isoform 1, novel protein similar to vertebrate receptor accessory protein family 5, and myozenin. The 28 upregulated ESTs also included genes with putative functions in the following three major categories: (1) transcriptional regulation (11%); (2) cell maintenance (11%); and (3) unknown (11%) (Table 4).

4. Discussion

Flavobacterium columnare is the causative agent of columnaris disease, causing annual losses of millions of dollars to the channel catfish industry in the United States. Aquavac-COL, a live attenuated *F. columnare* vaccine, has been developed to protect channel catfish from columnaris disease (Shoemaker et al., 2005, 2007). However, it is not clear how the catfish host responds to the vaccination soon after the immersion exposure to *F. columnare* vaccine. Therefore, the purpose of this study was to isolate upregulated genes in channel catfish fry soon after modified live *F. columnare* vaccination without any preconception of their identities. Using subtractive cDNA hybridization and qPCR, 28 ESTs representing 28 different genes were found to be upregulated by vaccination and the greatest portion (46%) of the upregulated genes were related to immune responses. For example, EST GW392458 was consistently upregulated greater than 3-fold in all three vaccinated catfish frys. GW392458 shared 64% identity with triglyceride lipase of Japanese eel (*Anguilla japonica*) at protein level (*e* value = 6E−37). The function of lipases is to hydrolyze phospholipids and triglycerides to generate fatty acids for energy production or for storage and to release inositol phosphates that act as second messengers. However, besides its central role in energy production, recent research has demonstrated that triglyceride-rich lipoprotein can bind to bacterial lipopolysaccharide (LPS) to protect against LPS-induced toxicity and modulate the overall host response to the LPS toxin (Parker et al., 1995; Barcia and Harris, 2005), suggesting that the overexpression of triglyceride lipases might be a specific innate immune response to modified live *F. columnare* vaccination.

Of all ESTs identified, EST GW392466 was upregulated the most (up to 256-fold) by modified live *F. columnare* vaccination. GW392466 shared 100% identity with intelectin 2 of channel catfish at protein level (*e* value = 5E−64). Intelectin is a soluble protein secreted into extracellular fluid which can induce agglutination, opsonization, or complement activation in host defense (Tsuji et al., 2001).

Table 3
Transcriptional levels of the 32 ESTs in *F. columnare* vaccinated catfish fry compared to that in non-vaccinated catfish fry.

EST clone no.	Accession no.[a]	Putative protein	Relative transcriptional level in vaccinated fish compared to non-vaccinated fish ($2^{\Delta\Delta C_t}$/fold)		
			$2^{\Delta\Delta C_t-1}$	$2^{\Delta\Delta C_t-2}$	$2^{\Delta\Delta C_t-3}$
6	GW392457	Putative histone H3	2.7	21.1	4.5
9	GW392458	Triglyceride lipase	3.6	3.5	5.8
11	GW392459	Beta-actin	0.3	1.6	1.7
16	GW392460	Glyoxalase domain containing 4	2.2	18.5	4.2
17	GW392461	Fetuin-B	2.3	4.2	4.5
19	GW392462	Hemoglobin-beta	2.3	2.6	4.5
20	GW392463	Nascent polypeptide-associated complex subunit alpha	2.4	15.8	4.3
24	GW392464	Unknown	2.4	28.7	5.3
25	GW392465	La ribonucleoprotein domain family, member 1	1.3	2.4	0.9
33	GW392466	Intelectin 2	0.3	256.4	1.6
37	GW392467	Pyrophosphatase (inorganic) 1	2.3	36.8	4.1
38	GW392468	Polymerase (RNA) I polypeptide C	2.5	39.5	5.8
39	GW392469	Similar to CNOT1	4.0	67.5	4.8
41	GW392470	C1R/C1S subunit of Ca2 + -dependent complex	1.7	5.3	1.4
42	GW39247 1	PIKK family atypical protein kinase	3.0	4.4	4.3
45	GW392472	Membrane-spanning 4-domains subfamily A member 8A	1.5	7.3	0.2
46	GW392473	Splicing factor 3b, subunit 1 isoform 1	2.2	2.0	0.2
47	GW392474	Similar to Tyrosine aminotransferase	1.1	2.1	0.1
48	GW392475	Matrix metalloproteinase	2.0	67.0	1.4
50	GW392476	Novel protein similar to vertebrate receptor accessory protein family	2.4	2.7	0.6
56	GW392477	Unknown	1.7	16.5	0.2
67	GW392478	Myozenin	4.1	8.2	0.5
69	GW392479	CD59	3.0	5.3	2.2
70	GW392480	Sodium/potassium-transporting ATPase, alpha subunit	0.7	7.7	0.1
75	GW392481	Unknown	1.6	3.3	0.3
80	GW392482	RIO kinase 3	1.6	35.6	4.2
82	GW392483	Muscle creatine kinase b	1.6	3.4	0.7
83	GW392484	Actinin alpha 3	1.0	1.1	0.4
85	GW392485	Galectin-4	1.2	1.5	0.7
86	GW392486	Neurotoxin/C59/Ly-6-like protein	0.6	0.6	0.4
89	GW392487	Microtubule-associated protein, RP/EB family, member 2	1.8	3.3	1.0
90	GW392488	Similar to myosin binding protein C, fast-type isoform 2	1.7	2.7	1.2

[a] EST accession number deposited at NCBI GenBank.

Intelectin can bind to arabinogalactan containing galactofuranosyl residues (Tsuji et al., 2001). Galactofuranosyl residues are contained in the cell walls of various microorganisms, including *Mycobacterium tuberculosis* (Daffe et al., 1990), *Streptococcus oralis* (Abeygunawardana et al., 1991), *Paracoccidioides brasiliensis* (Suzuki et al., 1997), and *Aspergillus fumigatus* (Leitao et al., 2003). It has been reported that intelectin 2 is upregulated 85-fold by *E. ictaluri* infection (Peatman et al., 2007). Similarly, substantial upregulation of intelectin-2 has been found in mice infected by nematode parasite *Trichinella spiralis* (Pemberton et al., 2004), suggesting that intelectin 2 may play an important protective role in the innate immune response of channel catfish to the modified live *F. columnare* vaccination. However, not all fish develop the same immune response to an infection at the molecular level. For example, we observed that intelectin 2 was upregulated 256-fold in only one of the vaccinated fish, suggesting that individual fish varies in immune responses to an infection or immunization. Wide variation of gene expression in individual fish has also been observed for 10 other genes in this study. Similarly, wide range of gene expression has been reported in individual zebra fish with or without infection by *Listonella anguillarum* (Rojo et al., 2007), suggesting that not all fish will have the same immune response/immunity to a vaccination or infection. Furthermore, individual fish might possess different degrees of immune system maturity although they are about the same age (7–10 days post-hatch), which might have contributed to the wide variation of gene expression for intellection 2 and some other genes in this study. Whether those genes are developmentally regulated merits further study.

Recently, 57 different genes have been isolated from a channel catfish subtractive library after *E. ictaluri* vaccination (Pridgeon et al., 2009a). However, in this study, only 32 ESTs were isolated from the *F. columnare* vaccinated channel catfish subtractive library. The difference between the numbers of ESTs identified from the two libraries might be mainly due to different sample collection time and differ-

Table 4
Putative function of the 28 *F. columnare* vaccination-upregulated genes.

Accession no.[a]	Protein	Putative function related to infection
Immune-related protein (13)		
GW392457	Putative histone H3	Innate immunity and cell signaling
GW392458	Triglyceride lipase	Innate immunity
GW392460	Glyoxalase domain containing 4	Protective immunity and pro-survival factor
GW392461	Fetuin-B	Acute inflammatory response
GW392462	Hemoglobin-beta	Immune protection against infection
GW392463	Nascent polypeptide-associated complex subunit alpha	Early immune response to infection
GW392466	Intelectin 2	Antimicrobial activity and protective role in innate immunity
GW392467	Pyrophosphatase (inorganic) 1	Protective immunity against infection
GW392470	C1R/C1S subunit of Ca2 + -dependent complex	Regulates phagosome maturation in macrophages
GW392472	Membrane-spanning 4-domains subfamily A member 8A	Enhances proinflammatory responses
GW392474	Similar to tyrosine aminotransferase	Acute infection response
GW392475	Matrix metalloproteinase	Proinflammatory and immune response
GW392479	CD59	Immune response to infection
Signal transduction (6)		
GW39247 1	PIKK family atypical protein kinase	Regulate DNA damage responses and nutrient-dependent signaling
GW392482	RIO kinase 3 (RIOK3)	Cell signaling (overexpression of RIOK3 inhibits TNF alpha-induced NF-kappaB activation)
GW392483	Muscle creatine kinase b	Cell signaling and regulation of T Cell development and activation
GW392473	Splicing factor 3b, subunit 1 isoform 1	Cell signaling (substrate of mitogen-activated protein kinase)
GW392476	Novel protein similar to vertebrate receptor accessory protein family 5	Regulates cell growth and membrane traffic
GW392478	Myozenin	Bridges Ca^{+2}/calmodulin-regulated protein phosphatase calcineurin to actinin
Transcriptional regulation (3)		
GW392469	Similar to CCR4-NOT transcription complex subunit 1/CNOT1	Global regulator of gene expression
GW392465	La ribonucleoprotein domain family, member 1	RNA binding
GW392468	Polymerase (RNA) I polypeptide C	Transcription
Cell maintenance (3)		
GW392480	Sodium/potassium-transporting ATPase, alpha subunit	Fluid homeostasis
GW392487	Microtubule-associated protein, RP/EB family, member 2	Inhibition of mitosis
GW392488	Similar to myosin binding protein C, fast-type isoform 2	Muscle contraction
Unknown (3)		
GW392464	Unknown	Unknown
GW392477	Unknown	Unknown
GW392481	Unknown	Unknown

[a] EST accession number deposited at NCBI GenBank.

ent protection mechanisms of the two different modified live vaccines. In the *E. ictaluri* vaccinated catfish subtractive library (Pridgeon et al., 2009a), we collected samples at 48 h post-vaccination. However, we collected samples at 10 min post-vaccination in the present study. We chose 10 min because that is the duration within which catfish frys are exposed to the vaccine at the full dose, thus the most stressful span of time for them. The 28 ESTs identified from this study represent genes with putative functions mainly associated with immune response (46%) and signal transduction (21%), whereas the 43 ESTs from the 48 h post-*E. ictaluri* vaccination (Pridgeon et al., 2009a) were genes with putative functions mainly in immune response (28%) and metabolism (21%). The identification of immune response-related genes after modified live vaccination of either *E. ictaluri* or *F. columnare* in channel catfish is not surprising since the innate immune system is the first line of host defense for eliminating invading microorganisms. However, at 48 h post-vaccination by *E. ictaluri*, only 2% of genes identified were involved in signal transduction, whereas 21% of genes had putative functions in signal transduction at 10 min post-*F. columarne* vaccination, suggesting that signal transduction plays an important role in the initial process of host defense system to sense the infection. It is not surprising to find the upregulation of immune response genes within 10 min after vaccination because it has been reported that large numbers of proinflammatory genes associated with the innate immune response are upregulated within 30 min after LPS challenge in the lungs of mice (Thimmulappa et al., 2006). Early signaling events have also been reported in host responses to infection. For example, it has been reported that the invasion of

Rickettsia conorii (the causative Gram-negative bacterium of Mediterranean spotted fever) into mammalian cells is dependent on phosphoinositide (PI) 3-kinase and on protein tyrosine kinase (PTK) activities. The internalization of *R. conorii* has been reported to be correlated with the tyrosine phosphorylation of several other host proteins, including focal adhesion kinase (FAK), within minutes of *R. conorii* infection (Martinez and Cossart, 2004), suggesting that the upregulation of three kinases (PIKK family atypical protein kinase, RIO kinase 3, and muscle creatine kinase b) might be a specific manifestation of host responses to *F. columnare* vaccination in the channel catfish.

In this study, EST GW392475 was upregulated as high as 67-fold by the modified live *F. columnare* vaccination. GW392475 shared 60% identities with matrix metalloproteinase of *O. mykiss* (GenBank protein NP_001117671, *e* value = 2E−34). It also shared 58% identities with collagenase 3 of *S. salar* (GenBank protein NP_001133996, *e* value = 2E−32). In our previous study, a matrix metalloproteinase 9 (MMP-9) was identified to be upregulated as high as 27-fold by modified live *E. ictaluri* vaccination (Pridgeon et al., 2009a). The MMPs are a family of zinc-containing enzymes with proteolytic activity against a wide range of extracellular proteins (Brinckerhoff and Matrisian, 2002). MMPs are expressed in a variety of normal and disease processes, such as development, involution, repair, inflammation, and tumor growth. MMPs have historically been thought to mediate remodeling or destruction of structural components. However, studies with genetically modified mice have demonstrated that MMPs have predominant roles in controlling the activity of effector proteins that function in immune processes (Parks et al., 2004). It has been demonstrated that LPS, the major constituent of the outer cell wall of Gram-negative bacteria and the principal mediator of inflammatory responses to these pathogens, is able to induce the release of MMP by neutrophils and monocytes in vitro (Masure et al., 1991; Opdenakker et al., 1991). Moreover, in mice, *E. coli* LPS administration has led to a quick release of MMP into the circulation, with peak values as soon as 1 h after injection (Dubois et al., 2002), suggesting that the upregulation of GW392475 in the channel catfish might be specifically induced by *F. columnare* vaccination.

In summary, 32 different genes were isolated from *F. columnare* vaccinated vs. non-vaccinated channel catfish fry subtractive cDNA library. Of the 32 ESTs, 12 were consistently induced at least 2-fold higher in vaccinated fish compared to unvaccinated control fish. Of the 12 upregulated genes, three (triglyceride lipase, PIKK family atypical protein kinase, and CCR4-NOT transcription complex subunit 1) were consistently upregulated greater than 3-fold. The 12 consistently upregulated genes also included CD59, polymerase (RNA) I polypeptide C, pyrophosphatase (inorganic) 1, mannose-P-dolichol utilization defect 1, nascent polypeptide-associated complex subunit alpha, hemoglobin-beta, fetuin-B, glyoxalase domain containing 4, and putative histone H3. Our results suggest that subtractive cDNA hybridization and qPCR are powerful cost-effective techniques to identify differentially expressed genes in response to modified live *F. columnare* vaccination.

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